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Apoptosis: Giving Phosphatidylserine Recognition an Assist — with a Twist

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Recognition of exposed phosphatidylserine on apoptotic cells is critical for their removal. A recent study suggests that annexin I is also externalized on apoptotic cells and may enhance phagocytosis of dying cells through the phosphatidylserine receptor.

Clearance of dying cells is critical to tissue homeostasis in the multicellular organism. In recent years, accumulating data have suggested that removal of apoptotic cells is a two-stage process. First, cellular corpses are neatly packaged for recognition during the process of apoptosis. Their uptake by phagocytes (either macrophages or tissue cells) prevents the eventual release of potent proinflammatory intracellular contents, as well as intracellular materials that may be proimmunogenic. A second process involves the induction of an active anti-inflammatory response in the phagocyte following binding to the cellular corpse. Both the uptake and anti-inflammatory responses appear to require that the dying cell lose phospholipid asymmetry, thus exposing phosphatidylserine (PS) on the external leaflet of the plasma membrane [1,2]. Preventing loss of phospholipid asymmetry or blocking exposed PS with annexin V appears to abrogate both uptake of the cell corpse and release of anti-inflammatory mediators [1,3,4]. These two processes can be separated, however, because cells that engulf apoptotic cells inefficiently can still respond with a robust production of anti-inflammatory mediators. Moreover, specific stimulation of the PS receptor with an agonistic monoclonal antibody stimulates release of anti-inflammatory mediators, as does exposure to liposomes containing the L, but not D, stereoisomers of PS.

Many phagocyte receptors have been implicated in the removal of apoptotic cells, and in many cases the ligands they recognize are not known. Why there are so many receptors for removal and how these receptors interact with their targets is an active area of research. Adding a level of complexity, several bridging molecules linking specific receptors on the phagocyte to ligands on the dying cell appear to increase the efficiency of uptake. In many cases, these are known PS-binding proteins, such as the serum proteins β2 glycoprotein 1 (β2GP1) [5] and protein S (involved in anti-coagulation) [6], the growth arrest specific gene product GAS-6 [7], complement activation products [8] and the milk fat globule protein MFG-E8 [9]. In the cases of β2GP1 and protein S, the receptors on the phagocyte to which these proteins bind have not yet been identified. GAS-6 is a ligand for the receptor tyrosine kinase MER, reported to be involved in engulfment of apoptotic cells [10], whereas MFG-E8 is believed to bind to αvβ3 [9], the vitronectin receptor implicated in the recognition and uptake of apoptotic cells many years ago by Savill and colleagues [11]. A few years ago, Reutlingsperger posed the hypothesis that annexins might also serve to bridge apoptotic cells to hungry phagocytes (C. Reutlingsperger, personal communication). Now, a recent paper from Arur and colleagues [12] supports that hypothesis and adds a twist to the story: that annexin I may serve to facilitate or enhance the recognition of PS by the PS receptor.

Using a quantitative proteomics approach, Arur *et al.* [12] compared membranes from normal and apoptotic cells. They found that expression of annexin I protein was elevated in membranes from dying cells compared with viable cells. While no annexin I could be detected by immunofluorescence on the surface of viable cells, apoptotic cells appeared to express this protein in patches, reminiscent of the way in which exogenously applied annexin V binds to exposed PS on dying cells. Permeabilization of the cells showed that annexin I was recruited to the membrane early in apoptosis before its accumulation on the cell surface. These findings were confirmed by cell fractionation and western blotting. The translocation of annexin I appeared to require activation of caspases as well as an increase in intracellular calcium levels, but the mechanism by which the protein is translocated across the membrane remains mysterious. It is interesting to note, however, that the ATP-binding cassette transporter A1 (ABC A1), implicated in the recognition and uptake of apoptotic cells by macrophages [13], has also been linked to the externalization of annexin I in pituitary folliculo-stellate cells [14].

A role for annexin I in uptake was confirmed by the use of small interfering RNAs to reduce annexin I expression in the dying cells. The efficiency of engulfment of these cells was reduced by approximately 60%. Uptake of these annexin I-silenced apoptotic cells was restored by adding exogenous annexin I, further supporting a role for this protein in clearance. Downregulation of the expression of the nematode annexin I homologue, nex-I, impaired engulfment of apoptotic cellular corpses in *C. elegans* as well, supporting the idea of phylogenetic conservation of this uptake pathway.

Following these data supporting a role for annexin I bridging PS on apoptotic cells to phagocytes, Arur and colleagues [12] then closed the loop by suggesting involvement of the PS receptor in this uptake pathway. Immunofluorescence studies revealed that the PS receptor on phagocytes appeared to cluster around the dying cell; this clustering was reduced when the apoptotic cells had been silenced for annexin I expression using siRNA.

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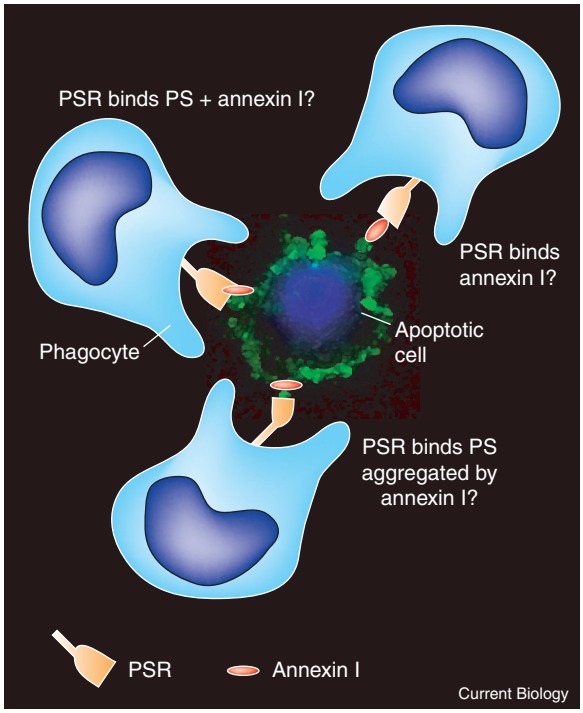

Figure 1. Three possibilities for interaction of annexin I with phosphatidylserine receptor (PSR).

Annexin I is represented in red. An apoptotic cell stained with annexin V (green) is surrounded by potential phagocytes. The PSR (orange) might bind to exposed PS that is configured in a recognizable 'eat-me' form by annexin I (bottom). Alternatively, PSR might bind to a ligand composed of PS and annexin I (upper left) or PSR might bind annexin I, which serves as a bridge between exposed PS on the dying cell and PSR on the phagocyte (upper right).

These interesting findings pose several questions. First, what does the PS receptor see on apoptotic cells? There is evidence that this PS receptor binds preferentially to PS compared with other phospholipids when coated on plates in an ELISA assay (V.A.F. and D. Xue, unpublished data) in the absence of any added protein. It is not clear how PS in an apoptotic cell membrane is recognised by PSR on the phagocytic cell. Several of us have wondered how such a small receptor molecule recognizes such an exceedingly small polar head group on a membrane phospholipid. Nevertheless, is there a 'missing link' that facilitates the presentation of PS in an as yet unknown configuration to the PS receptor on the phagocytes?

One could speculate that a membrane-associated protein or proteins might enhance the physical array of PS so that it is recognizable to a phagocyte as an 'eat me' signal. If true, the existence of such a protein might be one of the explanations for why activated cells, which express PS transiently, are not engulfed by phagocytes. Is it possible that annexin I is the missing link? Is the true ligand for the PS receptor annexin I *plus* PS? Or could the PS receptor be associated with the annexin I/lipocortin receptor proposed several years ago? If so, the distinctive stereospecificity of the PSR, in contrast to many other lipid-binding receptors, has to be explained. The molecular interactions among these three molecules requires elucidation (see Figure 1). More likely, one might speculate that annexin I is somehow involved in the movement of PS from the inner to the outer leaflet of the cell during apoptosis or that it aggregates PS in the outer leaflet without necessarily bridging PS to PSR. Does PS appear aggregated in annexin I deficient cells as it does in wild-type cells?

Given that the PS receptor is required for engulfment of apoptotic cells as well as the release of anti-inflammatory mediators associated with binding to apoptotic cells, and given that the anti-inflammatory effects of apoptotic cells can be mimicked with protein-free liposomes containing L, but not D stereoisomers of PS, is annexin I important only for efficiency of engulfment? Or does it also facilitate efficiency of the anti-inflammatory response? Annexin I (originally designated lipocortin) has a long history as an anti-inflammatory molecule induced by glucocorticoids. Originally believed to be an inhibitor of phospholipases, it is now believed to inhibit the function of these enzymes by binding to PS, and preventing its interaction as cofactor with the phospholipase. Furthermore, annexin I action is not restricted to the inhibition of phospholipase activity; it is known to inhibit a variety of inflammatory pathways. It will therefore be critical to determine if silencing annexin I also blocks the anti-inflammatory response induced by apoptotic cells.

A third question concerns how annexin I bound to the external surface of apoptotic cells facilitates uptake in the face of the known ability of exogenously added annexin V to block uptake. The answer may relate to differences in thermodynamic properties between the two proteins contributing to different functions [15]. How PS in a cell membrane binds to both these annexins as well as how it binds to the PSR need to be clarified. Confirming a role for these molecules *in vivo* remains a critical goal.

Why are there so many PS-binding proteins that may be involved in phagocyte recognition of apoptotic cells? In general, the dogma is that apoptotic cells are anti-inflammatory. However, there are some scenarios in which they have been shown to be proinflammatory or proimmunogenic [16,17], particularly when used to stimulate immunity against tumours (for example, see [18–20]). Leaving out technical issues associated with purity of apoptotic cell populations and content of necrotic cells, these contrasting effects raise some interesting possibilities. Do some of the PS-binding proteins in fact block the anti-inflammatory effects of PS? If so, the true effects of the apoptotic cell on inflammation and immune response will be determined by how much PS is bound by these various proteins *in vivo* versus how much is free, and whether these proteins trigger anti-inflammatory or proinflammatory receptors on phagocytes.

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